

Somatic Mosaicism and Cancer: A Micro-Genetic Examination into the Role of the Androgen Receptor Gene in Prostate Cancer

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Abstract

Recent evidence has shown that the androgen receptor (AR) plays a major role in all prostate cancer stages, including both androgen-dependent and -independent tumors. A large number of studies have examined the possible effects of a functional polymorphism in the AR gene, a variable-length CAG repeat, on the development of prostate cancer, but the results to date have been inconclusive. We have considered the fact that the tissue heterogeneity present in almost all prostate cancer tumors has rarely been regarded as an indicator of AR genetic heterogeneity. To determine if genetic heterogeneity exists and is a significant event in prostate cancer development, we have examined prostate cancer tumors for somatic shortening of the AR gene CAG repeat. All 72 laser capture microdissected samples from archival prostate cancer tissues, as well as samples from freshly prepared prostate cancer tissues, showed some genetic heterogeneity (somatic mosaicism) for AR CAG repeat length. Cancerous tissues showed a much greater degree of genetic heterogeneity than adjacent benign tissues, as well as a very significant shortening of their CAG repeat lengths. However, CAG repeat length heterogeneity was not observed in normal prostate tissues. It is hypothesized that somatic mosaicism of the AR CAG repeat in prostate cancer tumors may be found to be an important genetic event in precancerous tissue, which may subsequently lead to the development of prostate cancer. (Cancer Res 2005; 65(18): 8514-8)

Introduction

Tissue heterogeneity, which is a property of almost all tumors, has rarely been considered to be an indicator of genetic heterogeneity. The significance of possible genetic heterogeneity in the development of cancer has been noted in a recent review (1), but until very recently, the microgenetic analysis (genetic analysis of microscopically distinguished tissues) required for such a study has not been technically feasible. The development of laser capture microdissection (LCM) has now made microgenetic analysis possible (2-4). We have used LCM to examine the microgenetics of prostate cancer.

Prostate cancer is the most common male malignancy in the Western world. Androgens are the single largest risk factor in

determining the incidence of prostate cancer, in that androgen deficiency lowers risk whereas androgen excess raises risk (5). In prostate cancer patients, the change from androgen sensitivity to insensitivity of prostate cancer tissues is of greatest significance in determining patients' ultimate prognosis, as androgen-insensitive (independent) prostate cancer is associated with a much poorer prognosis than androgen-sensitive (dependent) prostate cancer (6). Of particular importance is that androgen receptor (AR) expression is maintained throughout prostate cancer progression and that the majority of androgen-independent prostate cancers continue to express AR (7).

The AR is a member of the nuclear receptor superfamily and is a ligand-dependent transcription factor. The AR gene is characterized by a polymorphic CAG repeat that codes for a glutamine tract whose length normally varies from 10 to 36 repeats, with 95% of individuals having 16 to 29 repeats (8). Variation in the AR CAG repeat length has been shown to be functionally significant in that it is inversely related to AR transcriptional activation (9). Further, a mechanistic relationship has been shown between AR polyglutamine (polyGln) tract truncation and androgen-dependent transcriptional hyperactivity in prostate cancer cells (10). Therefore, it is not surprising that a number of studies have shown that shorter-length AR CAG repeats could be considered a possible risk factor for prostate cancer (7). Overall, however, more than 20 primary epidemiologic studies have as yet failed to confirm a consistent and reproducible association between germ line AR CAG repeat length and prostate cancer (11), and a recent analysis of the data from all such reported studies has shown an overall shortening of less than one repeat length (12). In addition, it is important to note that almost all these germ line studies have examined AR CAG repeat length in blood leukocytes, cells that are not known to be androgen responsive, rather than in actual prostate tumor tissue.

In one of the first studies to examine AR CAG repeat length variation in actual tumor tissue, we examined CAG repeat length variation in breast cancer tumors and noted that in 14% of the tumors analyzed, an extra CAG repeat length was present (13), which is suggestive of somatic AR CAG length changes. Further, in a study of prostate cancer tumors (14), in one tumor the cancerous tissue had an extra CAG repeat compared with normal nonprostate tissue.

To more accurately assess if variation in CAG repeat length is associated with prostate cancer, a recent study examined AR CAG repeat lengths in prostate tumors using LCM and found *in situ* shortening of the CAG repeat length in both prostate cancer and its possible precursors (15). Particularly intriguing was that in 26% of the cases, two different repeat lengths were found, effectively demonstrating somatic mosaicism. In another recent study, a similar observation was made in men with benign prostatic hyperplasia (16). Interestingly, somatic mosaicism has now been

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reported in a growing number of genetic diseases (17) and has been shown to be a primary source of variable expressivity (18). Finally, the fact that these are somatic gene alterations is important, as it has been observed that somatic mutations are significantly more important in transforming normal cells into cancer tissues than germ line mutations (19).

It has been noted that the instability of the *AR* CAG repeats in sperm cells is 10^4 greater than would be expected based on normal mutation rates (20). The possibility that this instability is a significant event in development of prostate cancer has resulted in the present study. We have examined the nature and degree of the *AR* CAG repeat instability and considered whether it can be a factor associated with early focal hyperplasia and/or prostatic intraepithelial neoplasia, as well as the subsequent development of these tissues into malignant prostatic tumors.

Materials and Methods

Tissue samples. Study material was drawn from radical prostatectomy specimens from untreated patients diagnosed with prostate cancer. The prostates were fixed in formalin, prosected as whole mounts, and embedded in paraffin blocks. Five-micrometer-thick sections were cut from each paraffin block before mounting on glass slides and staining by H&E. Cancers were diagnosed using standard pathologic criteria and graded and staged according to the Gleason and tumor-node-metastasis systems, respectively.

Laser capture microdissection. Histologic sections were prepared as above. Under microscopic visualization, areas of interest in the sectioned tissue were mapped for LCM. Microdissection of each area was done using a PixCell II LCM system (Arcturus) according to standard methods using the finest laser setting (7 mm). Approximately 2,500 to 4,000 cells were microdissected per sample, and a total of 78 samples were collected. For each sample, the melted film containing the target cells was incubated in 50 μ L of lysis buffer containing 10 mmol/L Tris (pH 8.0), 2 mmol/L EDTA, 0.2% Tween 20, and 200 μ g/mL proteinase K at 65°C for 16 hours. The samples were then heated at 95°C for 10 minutes to inactivate the proteinase K. Five-microliter aliquots were used for PCR and the remainder of the DNA samples was kept at -20°C.

PCR. The CAG repeat was amplified by two rounds of PCR using nested primers flanking the CAG repeat in exon 1. The primers used were, for outside primers, primer 1.1A: 5' GTG GAA GAT TCA GCC AAG CT 3' and primer 1.1B: 5' TTG CTG TTC CTC ATC CAG GA 3'; for inside primers, primer 1.15A: 5' ACC CAG AGG CCG CGA GCG CAG 3' and primer 1.15B: 5' TGG GGC CTC TAC GAT GGG CTT 3'.

The CAG repeat was amplified using HotStarTaq DNA polymerase (Qiagen, Chatsworth, CA) in combination with Q-solution for higher fidelity PCR of GC-rich templates. A 50 μ L aliquot of reaction mixture containing 5 μ L of microdissected solution, 100 ng of primers 1.1A and 1.1B, 0.15 mmol/L of each deoxynucleotide triphosphate, 1 \times HotStarTaq amplification buffer,

1 \times Q-solution, and 3.75 units of HotStarTaq DNA polymerase was amplified by successive rounds of PCR. The conditions for the first round of PCR were as follows: 95°C for 15 minutes, then 40 cycles (94°C \times 30 seconds, 58°C \times 45 seconds, 72°C \times 45 seconds), and a final elongation at 72°C for 5 minutes. The second PCR used 5 μ L of the first PCR reaction, primers 1.15A and 1.15B, and the same reaction conditions as the first PCR. The number of PCR cycles is similar to that of a number of other studies in which genes from microdissected tissues were sequenced (21, 22). All the PCR reactions were prepared in a PCR workstation to prevent contamination.

PCR products from the second round were electrophoresed in 2% agarose gels and visualized with ethidium bromide. The bands of interest were excised from the gel using a clean blade and the DNA extracted and purified using the MinElute Gel Extraction kit (Qiagen).

Sequencing. Sequencing of the CAG tract was carried out using the ThermoSequenase Radiolabeled Terminator Cycle kit (Amersham Biosciences, Baie d'Urfe, Quebec, Canada). Samples were prepared following an optimized protocol using a dITP mastermix and loaded onto a sequencing gel prepared using a Gene-Gel 6% gel-casting solution (Bioshop Canada, Inc., Burlington, ON, Canada). The gel was vacuum dried and exposed to Kodak Biomax MR film (Eastman Kodak Company, Rochester, NY) overnight at room temperature. All samples were sequenced three separate times to ensure that the results were reproducible.

Results

Patient data. The present study examined prostate tissue from five males of ages 57 to 70 years who had been diagnosed with early-stage prostate cancer (Table 1).

All had elevated prostate-specific antigen levels and had been treated with radical surgery but not hormones. Two of five had symptoms and four of five had multinodular disease. A pathologist (K.S.) examined selections of previously prepared sections, before LCM, and extensively mapped and pathologically graded specific areas of prostate tissue (Fig. 1A). These areas were reexamined after LCM to ensure that the initially identified tissues had been correctly microdissected. Specific areas included either benign or cancerous tissue of various grades (according to their Gleason scores) and contained a mixture of stromal and epithelial tissue.

Different *AR* CAG repeat lengths found in laser capture microdissected samples. After microdissection, DNA was extracted from the blinded samples, and their *AR* CAG repeats sequenced several times. Surprisingly, the microdissected samples showed a number of different CAG repeat lengths (Fig. 1B). This genetic heterogeneity was found in all of the patient samples examined (Fig. 2A).

Somatic mosaicism of *AR* CAG repeat length identified in prostate cancer samples. In areas identified as cancerous (i.e., with a Gleason grade of 3 or more), there was always at least one

Table 1. Patient data

Patient	Age	PSA	PSA/prostate volume	Gleason score	Gleason pattern	PIN	HGPIN	Cancer zones
A	63	20.0	0.46	8	4 + 4	×	✓	PZ, TZ
B	61	40.0	1.18	7	3 + 4	✓	✓	PZ, CZ
C	70	10.7	0.27	9	5 + 4	×	✓	PZ, TZ
D	67	11.4	0.15	5	3 + 2	×	✓	PZ, TZ
E	57	13.5	0.61	5	2 + 3	×	✓	PZ, TZ

Abbreviations: PSA, prostate-specific antigen; PIN, prostatic intraepithelial neoplasia; HGPIN, high-grade prostatic intraepithelial neoplasia; PZ, peripheral zone; CZ, central zone; TZ, transitional zone.

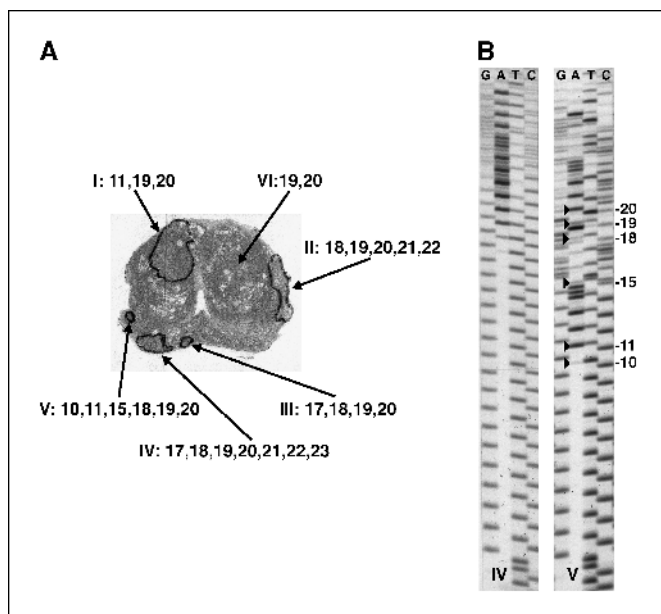


Figure 1. A, LCM and *AR* CAG repeat sequencing of prostate cancer tissue. Each of the outlined areas has been identified as consisting of homogeneous cancer tissue. Areas I to V were classified with a Gleason grade of 3. Tissue taken from area VI was classified as benign. The numbers following each area are the different lengths of *AR* CAG repeats found following sequencing of the microdissected areas. B, sequencing gels of areas IV and V (Fig. 1A). The CAG repeat was sequenced in the reverse sense and thus appears as a CTG repeat on the sequencing gel. The A nucleotides (arrows) identify the length of the trinucleotide repeats as shown at the right of the sequence.

repeat length of 18 or less (Fig. 2A and B). More surprisingly, even in areas of the tumor sections identified as histologically homogeneous, a number of different repeat lengths were identified (Fig. 2B), sometimes even below the extreme outliers (i.e., 10) of the reference range of CAG lengths (23), including some with zero CAGs (Fig. 2A). Such repeat lengths are entirely novel, as lengths this short have not been reported in normal individuals (8). In most cancerous tissues, many different lengths were found, often <15 and rarely >25, indicating a distinct trend towards significantly shorter CAG repeat lengths (Fig. 2B).

Further, it was observed that high Gleason score tumors generally had shorter lengths, although any specific correlation must await a much larger survey that includes tumors from patients with late-stage disease. Significantly, areas identified as benign had many fewer different CAG repeat lengths, which only varied by one to three repeat lengths (Fig. 3). The occurrence of somatic mosaicism in all of the tumor tissues examined is highly significant and cannot be considered a random or serendipitous event.

To further validate the results, and to preclude any possible archival effects, we also examined fresh prostate cancer tissue. A number of fresh prostate cancer tumor tissue samples were subjected to the same LCM-PCR sequencing protocol and were found to have similar CAG repeat length variation both for cancerous and benign tissues (Fig. 4A). As an additional control, we were fortunate to obtain tissue from a normal prostate and found only one repeat length in samples from three different zones (Fig. 4B). In addition, it should be noted that in the study by Tayeb et al. (16), normal prostate epithelial tissue from men with benign prostatic hyperplasia had only a single CAG repeat length.

In light of the novelty of our observations, it was important to confront three issues. First, that the multiple CAG repeat lengths

observed were a PCR artifact. To validate our observations, we used simple plasmid DNA (*AR* cDNA) and several *AR* genomic DNA sources (e.g., genital skin fibroblasts, testicular tissue, and white blood samples as additional controls), and subjected all materials to the above PCR protocols. Importantly, all the starting DNA materials from the above sources were used in amounts equivalent to DNA from ~2,000 cells. In all these control cases, our sequence analysis did not show somatic instability of the *AR* CAG repeat, nor were short repeat lengths seen (data not shown). Further, hundreds of other individuals' *AR*s have been sequenced with similar results (24).

The second issue to be addressed was the possibility of PCR contamination. However, this was clearly not the case as very small CAG repeat lengths, which were clearly unique for each microdissected sample, were only ever found in those specific samples. The third issue is the possibility that the large number of CAG repeat lengths observed in prostate cancer tissue was an epiphenomenon due to the increase in growth and division of neoplastic and preneoplastic cells. However, this is highly unlikely as in previous studies of somatic alterations of the *AR* CAG repeat, both shortening and lengthening of the CAG repeat have been observed (20, 25). In the present study, however, a very distinct trend towards shortening of *AR* CAG repeat lengths was found in prostate tumor tissues.

Discussion

It is important to note that this study is different from previous studies that investigated the possible association of variation in the length of the *AR* CAG repeat with prostate cancer. Other studies have examined germ line tissues and then reported on the possible

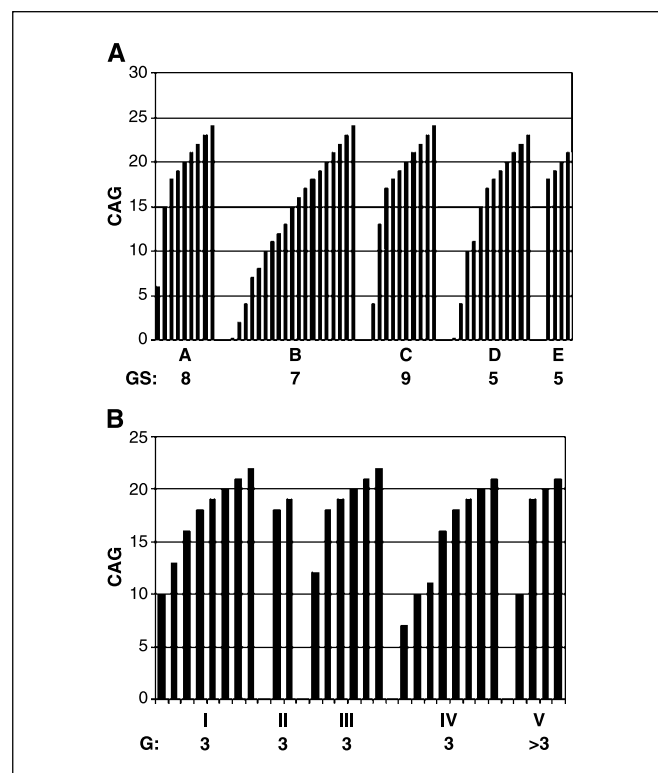


Figure 2. A, *AR* CAG repeat lengths found in LCM samples taken from all five prostate cancer patients. GS, overall Gleason score. Note that two patients (B and D) had an *AR* with no CAG repeat. B, samples taken solely from patient A. G, Gleason grade.

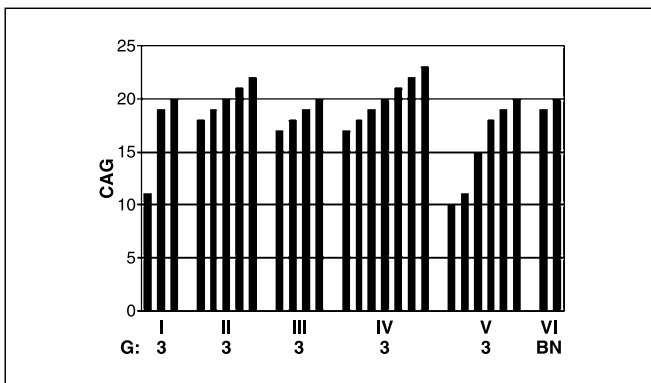


Figure 3. AR CAG repeat lengths from distinct LCM samples from patient D (areas I-VI). G, Gleason grade of each LCM sample. BN, an LCM sample taken from an area identified as benign.

significance of germ line shortening of the AR CAG repeat length in prostate cancer (12). In the tumor tissues we analyzed, the repeat length shortening and genetic mosaicism observed are somatic, rather than germ line, events. Further, as shown by sequencing DNA from microdissected tissues, these events occurred in over 70 tumor samples, whereas in previous germ line studies, the possible significance of germ line AR CAG repeat length shortening was solely based on statistical analyses. The other two studies that have examined prostate cancer tissue have also shown an *in situ* shortening of AR CAG repeat lengths but, in contrast to the present study, only observed somatic mosaicism in a limited number of their samples (15, 16). The most obvious explanation is that these researchers did not actually sequence the AR CAG repeats. Rather, sizing gels were used, which are likely to detect only large changes in CAG repeat length. This, in our experience, would make it very difficult, if not impossible, to detect all the different CAG repeat lengths present.

Knudson (26) noted that although over 30 mutant genes for hereditary cancers had been cloned in the past decade, penetrance depended on additional, somatic, mutations. A number of studies have discovered mutations in the AR in prostate cancer tissues (8). Particularly intriguing is that these mutations must result in a gain-of-function in the AR (27), in contrast to most AR mutations that result in a loss-of-function disease known as androgen insensitivity (24). The ability of an AR alteration to cause a gain of function in ARs is not unknown, as an increase in AR CAG repeats above 40 causes a motor neuron disease known as spinobulbar muscular atrophy (28).

Clearly, the identification of the events that result in the initial selection of cells containing AR mutations is of crucial importance. Recently, the functional significance of the length of the AR CAG repeat has been shown in prostate cancer cells. This study showed that AR proteins containing shorter polyGln tracts responded to lower concentrations of androgens than the wild-type AR (10). This would suggest that cells with shorter AR polyGln tracts are more likely to be selected (e.g., have a growth advantage) in androgen-responsive tissue. The accumulation of AR mutations in prostate tissue could thus be the result of both genomic instability and selection pressure.

One question that arises is why the vast majority of samples did not have zero CAGs if cells with zero CAGs have a selective advantage. However, it should be noted that although the rate of CAG shortening or lengthening is estimated to be 10^{-4} (20), this represents only single CAG repeat length changes. Thus, the

occurrence of zero CAGs is likely to be a fairly rare phenomenon and unlikely to occur in a majority of the tumor cells, and it would be highly unrealistic to expect a preferential selection for a majority of zero CAG length-containing cells.

It is known that almost all prostate cancer patients become resistant to therapy that blocks androgen-mediated cell proliferation (29). Initially, it was assumed that the AR would play no role in androgen resistance; however, recent studies have noted that an increase in AR mRNA and protein seems to be both necessary and sufficient to convert prostate cancer growth from a hormone-sensitive to a hormone-independent state (30, 31). In addition, AR CAG repeat lengths seem to be related to differences in the susceptibility to biochemical failure of postprostatectomy patients, which further supports the hypothesis that increased AR activity may indeed play a vital role in prostate cancer progression (32).

A recent study of mutant ARs from human prostate cancer tumors identified a significant number of the mutant receptors as promiscuous (i.e., transactivated by nonandrogens; ref. 27). This observation suggests a mechanism to explain the conversion of prostate cancer tissues to an androgen-insensitive state, particularly following antiandrogen therapy. Our study has revealed that some prostate cancer tissues completely lack AR CAG repeats (Fig. 2A). These cells would likely be more responsive to low androgen levels and thus likely to be preferentially selected in such environments. If the AR in such cells also contained mutations that caused the AR to bind nonandrogens, this could explain the progression of such cells to androgen independence.

This present study therefore proposes an additional step in the progression to androgen independence by suggesting that mutations are more likely to occur in genetically heterogeneous tissue that is differentially responsive to androgens.

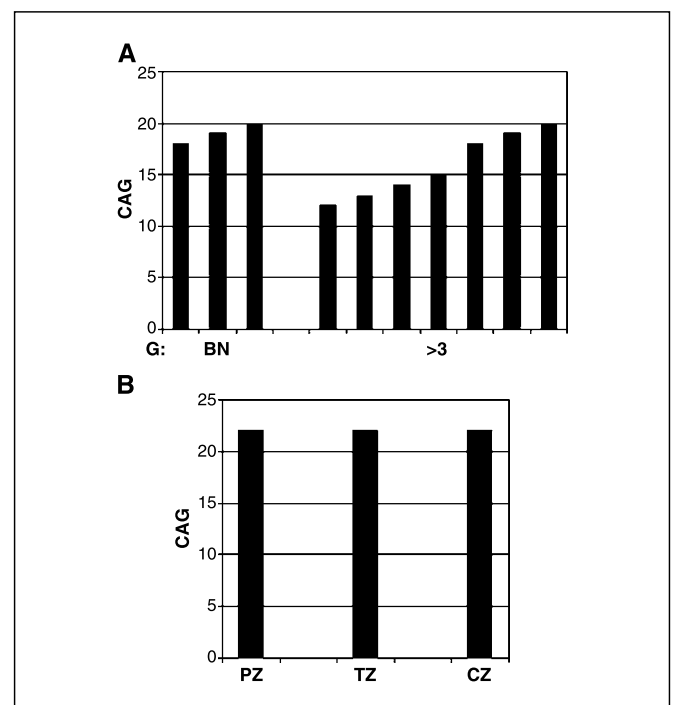


Figure 4. A, AR CAG repeat lengths sequenced from LCM samples from a fresh prostate tumor. G, Gleason grade. B, AR CAG repeat lengths sequenced from fresh LCM samples from a healthy prostate. PZ, peripheral zone; CZ, central zone; TZ, transitional zone.

In conclusion, we believe that this study is of particular significance in that somatic mosaicism of *AR* CAG repeats was found to be present in all tissues ranging from benign to cancerous, dissected from prostates removed from men diagnosed with prostate cancer. The fact that this mosaicism was observed in over 70 samples clearly distinguishes this observation, first, from the two previous studies that examined prostate cancer tumor tissue (15, 16), and second, from studies that have reported a possible association between shorter germ line *AR* CAG repeat lengths and prostate cancer (12). Thus, there may be possibly two risk factors associated with alterations in *AR* CAG repeat length: (a) an inherited germ line-specific repeat length and (b) the propensity of that inherited repeat length to undergo somatic shortening that results in somatic mosaicism.

We also believe that this study, which is the first to conduct a microgenetic analysis of prostate cancer by utilizing LCM on tumor tissues and then directly sequencing the *AR* CAG repeats from dissected cells, points the way to an approach that can much more accurately monitor and follow the crucial somatic events that can lead to both the initiation and progression of many types of cancer.

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